U.S. NAVAL
MEDICAL RESEARCH LABORATORY

Submarine Base, New London, Conn.

Vol. XXI, No. 2 REPORT NO. 371 5 January 1962

A CYTOCHEMICAL STUDY OF THE NEURONAL GOLGI APPARATUS IN EXPERIMENTAL DIPHTHERITIC ENCEPHALITIS IN RATS

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Research Project MR005.14-3002-8.03

SUMMARY PAGE

THE PROBLEM

To investigate the cytochemical alterations of the neuronal Golgi apparatus in toxic states (i.e. diphtheritic encephalitis induced in rats).

FINDINGS

The neuronal Golgi apparatus becomes fragmented and lost within 24-48 hours after administration of diphtheria toxin.

APPLICATION

The alterations of the Golgi apparatus in diphtheritic encephalitis suggests its usefulness as an index of early neuronal damage in central nervous system toxicology.

ADMINISTRATIVE INFORMATION

This investigation was undertaken as a part of Bureau of Medicine and Surgery Research Task MR005.14-3100-8, A Study of Alterations in Cytological Organelles as an Index of Early Nerve Cell Damage. The present report is No. 3 on this Subtask,—the two previous reports being NMRL Numbers 362 and 367 (1961).

Reprinted from the Archives of Neurology
November 1961, Vol. 5, pp. 497-503

A Cytochemical Study of the Neuronal Golgi Apparatus

Experimental Diphtheritic Encephalitis in Rats

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Further investigations of enzyme activities in the rat brain that survive fixation in cold dilute formaldehyde [1] have demonstrated high levels of nucleosidediphosphatase activity in the Golgi apparatus of neurons.[2,3] The classical metal impregnation methods for staining the Golgi apparatus are generally tedious and fickle, and they preclude parallel enzymological studies.[4] The method used in this study [3] rapidly visualizes the Golgi apparatus in frozen sections and permits cytochemical studies of other cell organelles in the same or parallel sections.[5]

It has been shown that acid phophatase-rich granules (lysosomes) undergo changes early in the course of neuronal necrobiosis produced by anoxia [6] and diphtheria toxin.[7] With the availability of a cytochemical method for demonstrating the Golgi apparatus and its nucleosidediphosphatase activity, it is now possible to compare alterations in these 2 related organelles during neuronal pathology.

This paper describes the neuronal Golgi apparatus, as seen in nucleosidediphosphatase preparations, and illustrates the applicability of these cytochemical methods in neuropathology by a study of diphtheritic encephalitis in rats. The observations on the nucleosidediphosphatase activity constitute the first step in mapping the brain for an enzyme localized in the Golgi apparatus, an organelle reaching extraordinary development in most neurons.

Methods

Male and female Sprague-Dawley rats weighing 150-300 gm. were used. They were killed by crushing of the cervicothoracic spinal cord; the cranial vault was rapidly reflected and the intact brain removed. The brain was divided into 2-3 mm. thick coronal and longitudinal sections and placed in ice-cold formol-calcium.[8] Twenty normal rat brains were processed for examination of lysosomes and Golgi apparatus.

Pathological alterations in the neuronal Golgi apparatus and lysosomes were demonstrated in the

Received for publication June 19, 1961.

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extensive lesions produced by the intracerebral injection of 0.01 cc. diphtheria toxin.[9] Each of two groups of 24 rats was given either 500-1,000 guinea pig minimum lethal dose (M.L.D.) or 50 guinea pig M.L.D. of purified toxin.* They were killed 6-36 hours and 12-72 hours, respectively to the group, after inoculation. Six control rats, given an intracerebral injection of 0.01 cc. isotonic saline, were killed after 6, 12, and 48 hours. In all cases, sections contralateral to the site of injection also were taken for study.

After fixation for 18-24 hours, the blocks were rinsed in cold water for 2-4 hours and placed in ice-cold 0.88M sucrose-1% gum acacia[10] for 24-48 hours to facilitate sectioning and storage. Frozen sections 10-15μ thick were cut with a Sartorius freezing microtome into ice-cold distilled water. These sections were stored in water at 2-4 C for 24 to 72 hours and then incubated for enzyme activity. No diminution in enzyme activity was noted as a result of this (and considerably longer) storage.

* Provided by Dr. C. G. Pope, Wellcome Research Laboratories, Beckenham, England.

Fig. 1.—Neocortex, layer VI. Nucleosidediphosphatase activity, with IDP as substrate. The well-stained Golgi lamellae (*G*) are pleomorphic. The capillaries (*V*) and glial fibers (*F*) are also stained. Reduced about 11% from mag. × 700.

Nucleosidediphosphatase activity was demonstrated by incubating sections for 20 minutes at 37 C in a modified Gomori medium containing inosinediphosphate (IDP) or thiamine pyrophosphate as substrate and manganese ions as activator.[8] Sections from the same block were stained for acid phosphatase-rich lysosomes by incubation for 90 minutes at 37 C in the glycerophosphate medium of Gomori.[11] In some instances the same sections were incubated first for acid phosphatase activity and then for nucleosidediphosphatase activity.

Control sections incubated in the absence of substrate were essentially negative in all instances.

Blocks of tissue adjacent to those taken for frozen sections were fixed in the same fashion, dehydrated in alcohols, imbedded in paraffin, and stained with hematoxylin-eosin and thionin ("paraffin Nissl").

Results

Normal Animals.—The Golgi apparatus is sharply delineated in the nucleosidedi-

Fig. 2.—Motor nucleus *NV*. Nucleosidediphosphatase activity, with IDP as substrate. The heavily stained Golgi lamellae (*G*) show a complex "reticular" nature. Arrow points to extensions in the dendrite. Glial fibers are seen at *F*. Reduced about 11% from mag. × 700.

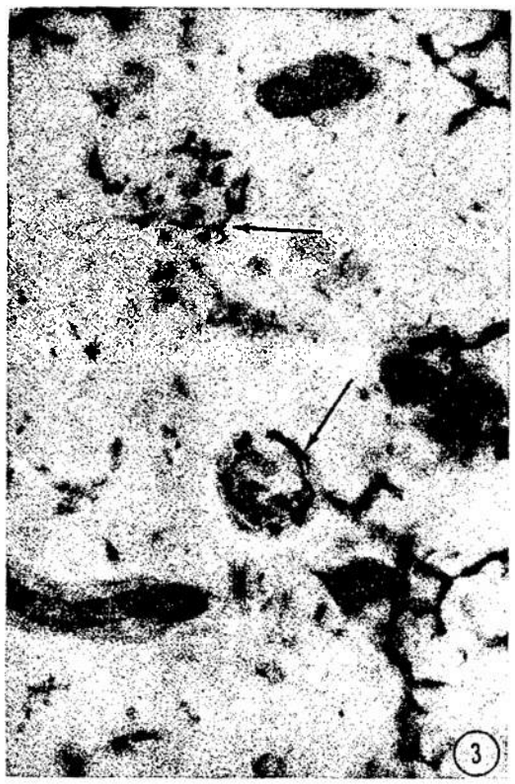

Fig. 3.—Purkinje cells. Nucleosidediphosphatase activity, with IDP as substrate. The elongated nature of the Golgi lamellae is evident only occasionally in this photograph (arrows). Most lamellae happen to be cut transversely, and thus appear ovoid. Reduced about 11% from mag. ×700.

phosphatase preparations (Figs. 1-3) because, as shown by electron microscopy,[3,5] in the neuron the reaction product of the enzyme reaction is restricted to the submicroscopic paired membranes[12-14] of this organelle. Under the light microscope, the aggregates of these membranes, covered with reaction product, are generally visualized as elongated, often branching and sometimes anastomosing, threads. More careful study shows the threads in these and other cells to be ribbons, or "lamellae."[12] In general, the larger neurons contain extensive and branching Golgi lamellae which stain intensely; the smaller neurons have less complex lamellae, which stain less intensely. Long and delicate lamellae are frequently seen extending into the dendritic processes, but are absent from the axons (Fig. 2).

The neuronal Golgi apparatus of the large motor neurons of the neocortex (Fig. 1), the hippocampus, Purkinje cells (Fig. 3), and cerebellar and cranial nerve nuclei (Fig. 2) are heavily stained. Those of the thalamus, hypothalamus, cerebellar granule cells, and remaining neocortex are moderately stained. The neurons of the basal ganglia and remaining brain-stem neurons are generally lightly stained.

It should be noted that within each architectonic zone considerable variations are present in both form and staining intensity of the Golgi apparatus. These variations are not due to technical factors, such as penetration of reagents, but reflect anatomical and probably functional differences among the neurons. Because they are the same in all untreated animals, departures from the normal in the Golgi lamellae of pathological tissue are readily detected.

The cell membranes of neuroglia, endothelial cells of capillaries, and smooth muscle of arterioles also split the substrates used to demonstrate the Golgi apparatus, as do the basement membranes of capillaries. Inosinediphosphate is readily hydrolyzed, while thiamine pyrophosphate is split slowly. Nucleosidetriphosphates, as previously reported with adenosinetriphosphaté, are also hydrolyzed by the cell membranes of blood vessels and neuroglia.

Even at short incubation times the cell membranes of the astrocytes stain so heavily for nucleosidephosphatase activity that sufficiently adequate resolution of the cell interior is difficult (Fig. 4*A*). The cell membranes of the oligodendroglia in the white matter are stained (Fig. 4*B*), while the perineuronal satellite cells of the gray matter are unstained. A few perinuclear filaments resembling Golgi lamellae are seen in some of the oligodendroglia.

The Golgi lamellae of the choroidal and ependymal cells appear as extensive delicate filaments lying near the luminal side of the nuclei.

The distribution of acid phosphatase activity in the lysosomes of the normal rat brain has been described in an earlier pub-

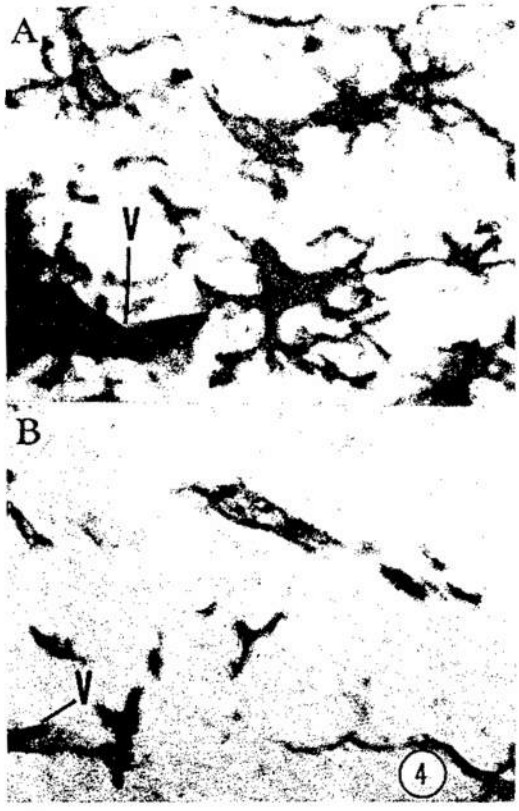

Fig. 4.—*A*, protoplasmic astrocytes. *B*, oligodendroglia. Nucleosidediphosphatase activity, with IDP as substrate. The glial staining is localized to the cell membranes. The membranes are difficult to resolve in the photograph. Capillaries (*V*) stain heavily. Reduced about 11% from mag. × 700.

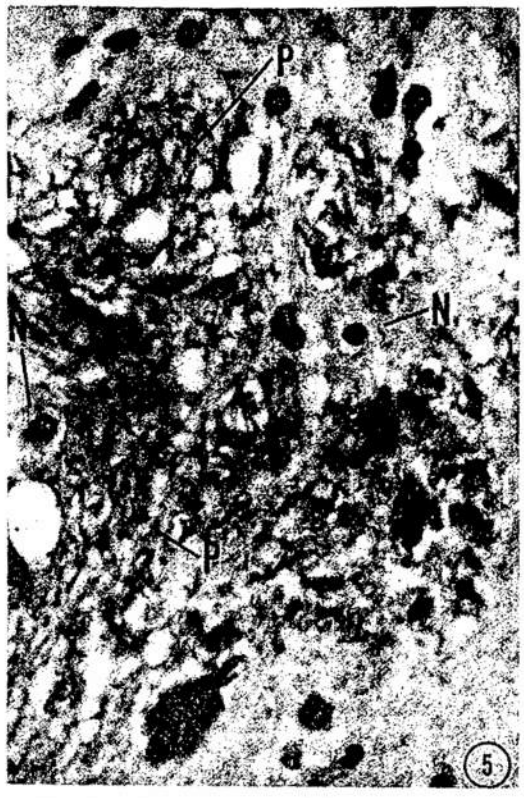

Fig. 5. — Neocortex. Diphtheritic encephalitis, 24 hours after 1,000 M.L.D. The neurons (*N*) are swollen or pyknotic and show variable staining. Spongy degeneration is present in the neuropil (*P*). Nissl stain; reduced about 11% from mag. × 200.

lication.[1] Staining of parallel or identical sections for acid phosphatase and nucleosidediphosphatase activities shows that many lysosomes lie very close to and upon the Golgi lamellae.

Diphtheritic Encephalitis.—The chronological sequence of clinical signs and the distribution of the neuronal lesions produced by the diphtheria toxin are similar to those originally described.[9] In the routine histological sections prepared from the 36 of the 48 animals that survived the procedure, there is evidence of neuronal degeneration and loss in all 12 rats permitted to survive 24 hours or longer (Fig. 5). Occasional zones of spongy necrosis and edema are evident in the neuropil. The Purkinje cells, granule cells, and the neurons of the neocortex are most frequently involved. Occasional lesions are seen in the basal ganglia, thalamus, and the cerebellar and brain-stem nuclei. The same distribution of the lesions is readily seen in the cytochemical preparations.

In the nucleosidediphosphatase preparations, no abnormalities are seen in the Golgi apparatus prior to 18 hours survival. On the other hand, swelling of the acid phosphatase-rich lysosomes ("cytolysome" formation) [15] is regularly seen 12 to 18 hours after both dosage levels (Fig. 8). Lysosomal swelling is most marked in the Purkinje cells and in the neocortex.

In the group given a 500-1,000 M.L.D. dose, a progressive loosening and loss of the Golgi lamellae appears after 18 hours. The altered lamellae that remain are rounded and show low levels of enzyme activity (Fig. 6). The loss of the fragmented

Fig. 6.—Purkinje cells, diphtheritic encephalitis, 18 hours after 500 M.L.D. Nucleosidediphosphatase activity, with IDP as substrate. The elements of the Golgi apparatus (*G*) are sparse and rounded and show little nucleosidediphosphatase activity. Reduced about 11% from mag. × 700.

Fig. 7.—Purkinje cells, diphtheritic encephalitis, 48 hours after 500 M.L.D. Nucleosidediphosphatase activity, with IDP as substrate. The remaining Golgi apparatus is fragmented into small spheres (*G*). Arrow points to a cell where virtually none of the Golgi apparatus remains. Reduced about 11% from mag. × 700.

lamellae is prominent after 24-36 hours survival (Fig. 7). In these late stages of degeneration, a few swollen lysosomes are seen, but most neurons show no acid phosphatase activity at all.

Similar cytochemical abnormalities in the Golgi apparatus occur in the 50 M.L.D. dose group, but do not appear before 24 hours and progress over a 72-hour period, at which time most of the enzyme activity is lost. Again, lysosomal changes precede those of the Golgi apparatus, and the changes in the Golgi apparatus are seen before the hematoxylin-eosin preparations show alterations.

In addition to the neuronal changes, there is in severe lesions a segmental interruption in the structure of capillaries and neuroglia. This is well visualized in the nucleosidediphosphatase preparations.

Comment

Cowdry [16] has summarized the results of early investigations in which classical metallic impregnation techniques were used in studies of pathological changes in neurons. The Golgi apparatus responds to varied conditions, such as lead intoxication, rabies, brain-stem transection, cold injury, transplantation, and autolysis with a common pattern—decrease in size and fragmentation ("retisolution"). In addition, a peripheral migration ("retispersion") of the Golgi fragments is observed during chromatolysis following axonal transection.[17] The fragmentation and dissolution of the Golgi apparatus in response to diphtheria toxin, identified by the present cytochemical method, resembles the classical observations ("retisolution").

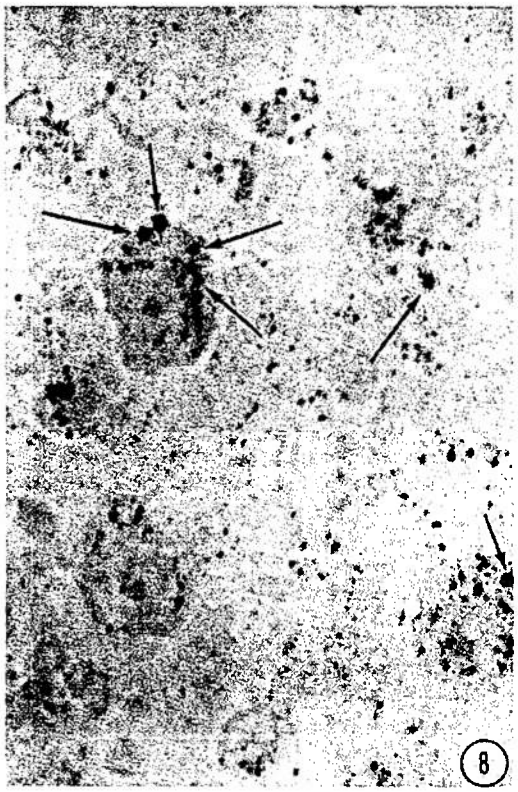

Fig. 8.—Purkinje cells, diphtheritic encephalitis, 12 hours after 500 M.L.D. Acid phosphatase activity, with glycerophosphate as substrate. Note the acid phosphatase-rich granules or lysosomes. Arrows point to some of the abnormal swollen lysosomes ("cytolysomes"). Reduced about 11% from mag. × 700.

The swelling of lysosomes prior to changes in the Golgi apparatus is consistent with the view that they may serve as sensitive indicators of early necrobiosis, in cells generally [15,18] and in neurons.[6,7]

The close spatial relation between many lysosomes and the Golgi lamellae that is observed in neurons is paralleled in many, but not all, cells.[5] This is consistent with the tentative suggestion that lysosomes, involved in pinocytosis or secrefion or both, may fuse with or arise from the Golgi "vacuoles." [18,19] Such speculations may be useful in suggesting studies with normal and pathological nervous tissue. Recently, Ogawa et al.[20] have concluded that the "neutral red granules" of cultured fibroblasts and astrocytes of cerebellar cortex are, in reality, lysosomes.

The availability of a simple, rapid, and reproducible means of demonstrating the Golgi apparatus in frozen sections may help reawaken interest of neurologists in this cell organelle. Its impressive size in neurons implies an important role, but this remains elusive. The finding of a specific nucleosidediphosphatase activity in the Golgi apparatus of neurons and all other cells tested[2,3] may suggest approaches to the investigation of its specific biochemistry. The ability to stain parallel or the same sections for activities of this specific phosphatase and for acid phosphatase activity associated with lysosomes may be used to elucidate the origin of neurosecretory granules. Electron micrographs suggest that secretory granules in many cell types,[12-14] including some neurosecretory granules of neurons,[21] arise in the Golgi apparatus. The granules appear in the spaces ("cisternae") enclosed within the paired membranes, and when they separate from the apparatus they are surrounded by Golgi membrane.

The ease of making such cytochemical preparations should enable the neuropathologist to study more readily the pathological alterations in the Golgi apparatus and to relate these to changes in other cell organelles such as lysosomes and mitochondria.

Summary

The distribution of nucleosidediphosphatase activity in the rat brain is described and its ability to visualize the neuronal Golgi apparatus is illustrated. Fragmentation and dissolution of the Golgi apparatus, as seen in the cytochemical preparations, is described in neurons injured by diphtheria toxin. Marked changes in the lysosomes occur before those in the Golgi apparatus.

The usefulness in neurology and neuropathology of the cytochemical staining procedures for the Golgi apparatus is briefly discussed.

The technical assistance of James F. Galvin HM1 and Miss Mary Catogas is acknowledged. We wish to thank Mr. George Dominguez for assistance with the photography.

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